Estimation of Gene Diversity at the *b* Locus of the Constant Region of the Immunoglobulin Light Chain in Natural Populations of European Rabbit (*Oryctolagus cuniculus*) in Portugal, Andalusia and on the Azorean Islands

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Manuscript received July 13, 1990
Accepted for publication November 7, 1990

ABSTRACT

The minimal gene diversity at a locus of the antibody constant region, as estimated in natural populations of rabbit, revealed levels of heterozygosity similar to those reported for the major histocompatibility complex in human and murine populations. Sera of 416 wild rabbits were collected on the Iberian peninsula and on three islands of the Azorean archipelago and analyzed for the occurrence of the serological markers of the *b* locus of the immunoglobulin light chain. All four serotypes present in domestic rabbits were found in Portugal. They represented less than 50% of the gene pool. In Andalusia this was less than 15% and on the Azorean islands less than 10%. The pronounced and systematic hierarchy in allele frequencies, previously found in populations from the more recent distribution area of the species, was not observed. On the peninsula, the frequencies of the "*domestic*" alleles were similar, averaging 10%. The Portuguese sample revealed a total heterozygosity of at least 87%. This high value was supported by at least 11 serologically different alleles, none of them occurring at frequencies above 20%. These data are in agreement with an Iberian origin of the European rabbit and strongly suggest the coalescence of *b* locus allelic lines drawn from Iberian and western populations. The role of balancing selection in the evolution of the *b* locus polymorphism was further emphasized.

IN European rabbits, the constant region of the light chain of the major immunoglobulin (Ig) fraction is controlled by the *b* locus (OUDIN 1960; reviewed by MAGE *et al.* 1973). In domestic breeds, four alleles are distinguished by current serological methods (the so-called *b4*, *b5*, *b6* and *b9* allotypes, which, for convenience, will be designated as *domestic* types). Although confirmed by direct genome analyses as true alleles of a single gene locus (EMORINE *et al.* 1984; MATTHIJSSENS *et al.* 1985), the constant regions of light chains of two *b* locus allotypes can differ at more than 30% of the 105 amino acid positions (BERNSTEIN, SKURLA and MAGE 1983). This is more interallelic divergence than observed at loci of the human or murine major histocompatibility complex (MHC), where more than 50 alleles are known to exist (KLEIN 1986). Observations on wild rabbits from Australia, Great Britain, Holland, Belgium and France (CURTAIN, WOOD and SOBEY 1977; VAN DER LOO *et al.* 1987; CAZENAVE *et al.* 1987) did not reveal the existence of *b* locus serotypes distinguishable from the four domestic ones. The domestic types occurred at frequencies showing a systematic hierarchy ($p_{b4} > p_{b5} > p_{b9}$; the *b6* allotype was observed only in France). However, studies on other leporid species (mainly *Sylvilagus* sp. and *Lepus* sp.) and on wild rabbits from Mediterranean regions (Northern Africa and the Iberian peninsula) revealed Ig light chain allotypes not found in domestic breeds and pointed toward the existence of a wide variety of *b* locus alleles (RODKEY 1972; VAN DER LOO, HAMERS-CASTERMANS and HAMERS 1976; LANDUCCI-TOSI, MAGE and TOSI 1976; SMITH and MANDY 1978; VAN DER LOO*et al.* 1980; BENAMMAR and CAZENAVE 1981, 1984; CAZENAVE *et al.* 1987).

In this paper we address two questions. One is about the history of the European rabbit species (*Oryctolagus cuniculus*), which is generally thought to originate from the Iberian peninsula. What are the gene frequencies of the *domestic b* locus alleles in this region and do these data support the hypothesis of an Iberian origin of the European rabbit? This is part of a more comprehensive genetic study on the European rabbit but should above all have implications for the estimation of allele coalescence times, *i.e.*, time at which sampled genes with a same allotype converge to a common ancestor (KINGMAN 1982). A second question concerns the degree of *b* locus heterozygosity in the putatively native range of the species and how it is distributed in comparison to the gene diversity in the more recently populated range.

The average heterozygosity and the number of alleles are, like the coalescence time, important parameters in the theoretical models developed to account for the unusual features of the polymorphism of MHC

loci (TAKAHATA 1990; TAKAHATA and NEI 1990). The estimation of these parameters for the *b* locus alleles could therefore be of more general interest as this polymorphism shares or even exaggerates some of the salient features of the MHC loci. Indeed, the coding regions of the C_{K1} gene of the *b4* and the *b5* allele differ at 39 nucleotide positions, of which 35 are associated with an amino acid replacement, while the associated untranslated regions and noncoding regions show 94–98% homology (BERNSTEIN, SKURLA and MAGE 1983; EMORINE *et al.* 1984; MATTHIJSSENS *et al.* 1985). Comparisons including the *b9* allele confirmed the four-to-one ratio of replacement versus synonymous substitutions (AKIMENKO, HEIDMANN and ROUGEON 1984; MAGE *et al.* 1987). This indicates that the extensive structural differences between allelic constant region of the rabbit Ig light chains resulted from an increase in rate of nonsynonymous nucleotide substitutions similar to that observed for the antigen binding regions of the MHC genes (HUGHES and NEI 1989). At the same time, population genetical analysis of alleles from the more recent distribution range of the species revealed highly significant non-random associations of alleles, suggesting strong overdominance and epistatic selection at the level of the *b* locus gene products (VAN DER LOO *et al.* 1987).

We report here the distribution and gene frequencies of the *domestic b* locus alleles in Portugal, Andalusia and on three Azorean islands and discuss how this can help to clarify the evolutionary patterns of this remarkable polymorphism. We also outline how an estimate of gene diversity in these areas can be obtained by using a panel of anti-allo antisera developed in domestic rabbits.

MATERIALS AND METHODS

Collection sites: Wild rabbits: *Portugal*: 189 specimen were from sites distributed over all major regions of Continental Portugal: Bragança (BR): 1 sample; Viana di Castelo (VI): 5 samples; Mealhada (ME): 7; Anadia (AN): 8; Leiria: 19; Idanha (ID): 11; Salvaterra (SA): 3; Santarm (ST): 21; Sesimbra (SS): 9; Bate-Orelhas (BO): 9; Vila Viçosa: (VV): 37; Mourâo (MO): 9; Reguengos de Monsaraz (RM) 33; Beja (BE): 7; Portimâo (PO): 5; Silves (SI): 5. *Andalusia* (Spain): 57 specimens from two localities within the vicinity of Laguna de la Janda (Cadiz province), 150 km South of Sevilla: Las Lomas P2 (L2): 38; P3 (L3): 19. *Azores*: 170 specimens from three islands: Flores (AF): 60; San Miguel (AM): 57; Tercheira (AT): 53. Domestic rabbits were obtained from local farms.

Sera: Blood samples were taken either from the marginal vein or from the heart for specimens that were trapped alive or from the thoracic cavity before *rigor mortis* for rabbits that were shot. Serum or plasma was separated and stored at −20°. Specimens were at least 3 months old, as judged by body size and weight.

Antisera: Antisera specific for the genetic markers of the *b* locus alleles ("anti-allotype antisera") were raised in rabbits with a particular *b* locus genotype against Ig from rabbits differing by one *b* locus allele but genetically identical for the Ig heavy chain markers (KELUS and GELL 1967; HAMERS-CASTERMANS *et al.* 1977).

Serological typing: Each serum was tested independently against anti-allo antisera specific for the allotypes *b4*, *b5*, *b6* and *b9*, by immunodiffusion in 1% agar gel containing 2% polyethylene glycol. In each test a reference was included to monitor the degree of serological similarity of the sample being tested with samples from rabbits expressing the immunizing allotype (see Figure 1). The reactions were recorded as type "1" in case of perfect fusion of the precipitation lines of test and reference sera with the antiserum ("*identity reactions*"), as type "*n*" in case the reference line spurs over the test line ("*crossreactions*," revealing serological similarity clearly distinguishable from identity reactions) and as "0" by negative test reactions. A serum from a heterozygous *b4/b9* rabbit will thus give rise to a pattern noted as (1001). The frequencies of the *domestic* alleles were estimated by considering the phenotypes (1*n*00), (100*n*) and so on, as heterozygous (*b4/bn* in this case). The probability that an (0100) pattern indicates homozygosity was inferred from the estimates of the relative frequencies of genotypes giving rise to (0*n*00) reactions.

The relative strength of the crossreactions (*n*) were, for each specificity, classified between 2 and 6 and subsequently grouped as *P* (2–3) or *W* (4–6). *P* corresponds to very clear crossreactions which were about as strong as or stronger than, the "spur" over the test reaction (*i.e.*, the precipitation of the reference with the antibody fraction not involved in the crossreaction with the serum being tested). *W* refers to weaker, less clear crossreactions (Figure 1). For the analysis of gene diversity, this last type of information was pooled with negative reactions (0). The resulting phenotypes define the smallest number of codominant, Mendelian alleles that would be sufficient to explain the observed situation. The likely gene frequencies were calculated using a reiteration procedure based upon binomial distribution of genes within localities, eventually searching for the best binomial fit.

Analysis of gene diversity or variance: The distribution of gene diversity was estimated following NEI (1973) and CHAKRABORTY (1974). H_T, the total heterozygosity in the sample, is the expected heterozygosity in a panmictic population with allele frequencies equal to the mean frequencies over localities. H_S is the average within-locality heterozygosity, H_R that within regions. D_{ST} (D_{RT}) is the diversity due to differences among localities (regions) and so on. We define further H_I as the heterozygosity within individuals and D_{IS} as the component of H_S which is due to variations within localities.

These components of diversity can be derived from:

$$H_T = 1 - \Sigma\, p_i^2$$

(where p^i is the mean frequency of the *i*th allele)

$$H_S = H_I + D_{IS}$$

$$H_R = H_S + D_{SR} = H_I + D_{IR}$$

$$H_T = H_R + D_{RT} = H_I + D_{IS} + D_{ST}$$

and can be related to the parameters of population structure as follows:

$$F_{ST} = 1 - H_S/H_T = D_{ST}/H_T$$

$$F_{IS} = 1 - H_I/H_S = D_{IS}/H_S$$

F_{IS} measures the local inbreeding coefficient while F_{ST} estimates the fraction of total diversity due to differentiation between subdivsions. The approximation method estimating

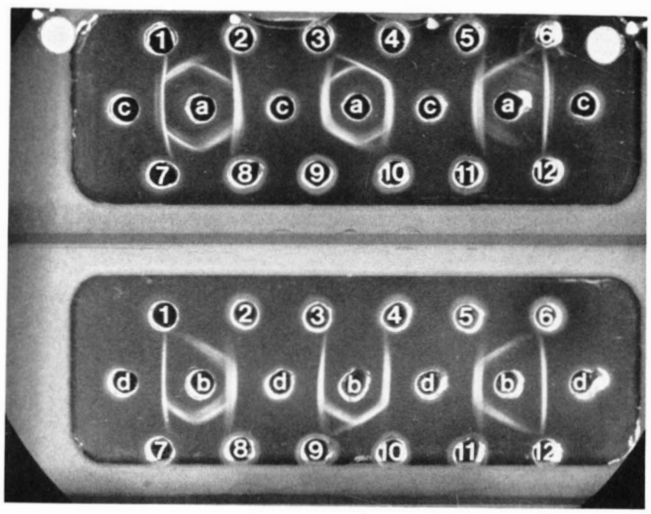

FIGURE 1.—Serological analyses of minimal *b* locus diversity by immunodiffusion. Allo-antisera specific for different *b* locus alleles were disposed into the center wells: *a*, anti-*b4* and *b*, anti-*b5*. Twelve sera from Portuguese wild rabbits were placed into the wells 1–12. Their precipitation reactions were evaluated against reference sera of the immunizing allotype: *c*, *b4* and *d*, *b5*. The weak *b4* reactions of sera in wells 6 and 11 are here ignored. We distinguish the following b4b5 phenotypes (see MATERIALS AND METHODS): **OO**, wells 6, 12; **OP**, well 11; **PO**, wells 1, 3; **PP**, wells 2, 5, 7, 8; **P1**, well 10; **10**, well 4; **1P**, well 9.

the genotype frequencies from the phenotype data searches solutions such that D_{IS} is minimal. Thus F_{IS} and D_{IS} are not estimates here.

Population parameters θ (correlation between genes from different individuals within subdivisions, which is analogous to F_{ST}) and its χ^2 statistics for $\theta = 0$ were also estimated for each allele, using the weighted procedure of COCKERHAM (1973). The mean of over alleles, θ_w, was estimated following REYNOLDS, WEIR and COCKERHAM (1983).

RESULTS

Estimating the frequencies of the *domestic b* locus alleles: All sera tested showed precipitation reactions with at least one of the antisera of the anti-*domestic* panel: if "*blank*" alleles existed, they were too rare to appear in the homozygote state in this study: *i.e.*, there was no (0000) phenotype. The vast majority of sera gave strong crossreactions with one or more types of antisera (Figure 1). However, for most of them the reference precipitation line spurs over the test reaction, which is a clear indication of genotypes different from the *domestic* ones (such spur implies that the test serum displays only a part of the epitopes recognized by the allo-antiserum on the Ig molecules of the reference allotype). This was very different from what we had found previously when the very same assay system was applied to wild rabbits from Australia, Kerguelen islands, Great Britain, and Continental Europe (North of the Pyrenean mountains) where all out of more than 4000 sera displayed either complete identity reactions or no crossreactivity at all with the *domestic* reference sera (VAN DER LOO 1987; W. VAN DER LOO and C. P. ARTHUR, unpublished results).

Only two out of the 416 sera analyzed here displayed no other reactivity than that which was recorded as *W*: they were discarded from the analysis. Identity reactions were observed, but none of the sera under study gave identity reactions with more than two types of antisera (Table 1). Of 33 sera showing identity for two different allotype specificities, 31 were completely negative for the two remaining allotypes (no *P* nor *W* reactions, see MATERIALS AND METHODS). These data were thus in agreement with the hypothesis that molecules giving rise to type "1" reaction (in general) do not crossreact with any of the other specificities (the two sera reacting with a third antisera are discussed below). Furthermore, identity reactions could be confirmed as such by distinct antisera of the same specificity. Thus, they revealed the presence of molecules serologically identical to the *domestic* allotypes. The frequencies of these *domestic* alleles were estimated by assuming binomial distribution within localities while considering any crossreactivity as a clear indication of the presence of an allotype different from the *domestic* ones (see MATERIALS AND METHODS). The estimates obtained were not significantly different when the weak crossreactions (type *W*) were ignored (see below). These results are listed in Table 2.

Two sera displayed a (1P10) phenotype. They occurred together in one sample locality (ST) where all "*b6*" identity reactions (. . 1 .) were associated with a strong "*b5*" crossreaction (. P1 .). This combination was also found elsewhere more often than expected. The existence of a *0P10* genotype was therefore postulated.

Estimate of the minimal number of common alleles: By considering for each specificity only the strong crossreactions (*P*) we distinguished the 33 phenotypes listed in Table 1. They can be explained by a minimum of 11 different genotypes. The binomial equation gave one solution when applied to the pooled sample from Portugal (187 rabbits). This frequency distribution was used at the start of the reiteration procedure for the estimation of allele frequencies in the subsamples and also in the Andalusian and Azorean samples (Table 2). The χ^2 statistics for the deviation from Hardy-Weinberg equilibrium are also shown.

Distribution of gene diversity and variance analysis: NEI's components of gene diversity are shown in Table 3 for the total sample. The data indicate an average heterozygosity of more than 80%. The largest fraction was contained within localities (and thus most likely within individuals) but a considerable fraction of the diversity (13–17%) was due to differences between localities. In Table 4 the gene diversity is shown for the three different regions separately. The mean

TABLE 1

Distribution of *b* locus phenotypes per locality

	Locality																					
Phenotype	AN	BE	BO	ID	LE	ME	MO	PO	RM	SA	ST	SS	SI	VI	BR	VV	L2	L3	AF	AS	AT	Total
0001									1							2						3
0010				1		1							1			4						7
0011		1		1			1					1				5						9
001P	1				1	1										2						5
00P0				1																		1
00P1				1						1												2
0100					1	2			2													5
0101							2		3													5
010P						1			1													2
0110						1	1	1	1							1						5
0P00				1	1				4	1	3			1		1	2	4	7	14	18	57
0P01									1	1		2		1			2					7
0P0P									1													1
0P10		1	1						4		1	2				1	1					11
1000					1		1				1					2		1		3	5	14
1001					1						1			1		3						6
100P	3															1						4
1010	3								1					1		1						6
1P00		1			1				1		3		1			2	1			4	9	23
1P10											2											2
P000			1					1								1	6		15	5		29
P001							1										1	1				3
P00P			1				1															2
P010								1					1			1	4					7
P0P0																1						1
P0P1																1						1
P0PP																1						1
P100			1		1	1		2	3						1	2						11
PP00		2	2	1	10		1		4		3	3	1			3	17	13	38	31	21	150
PP01					1				1		2	1		1			1					7
PP0P													1									1
PP10	1		2		1				2		5					1	3					15
PPP0		2		5			1		3													11
Sum	8	7	8	11	19	7	9	5	33	3	21	9	5	5	1	36	38	19	60	57	53	414

At least 32 different serological phenotypes were clearly distinguished by immunoprecipitation reactions using four antisera of different specificity. "1" indicates serological identity reactions, "P" strong to very strong crossreactions, "0" weaker crossreactions or negative reactions.

values of H_S and H_I over regions are shown in Table 2 or can easily be derived from it. The Portuguese sample scored with $H_R = 0.88$ the highest diversity per region. It was also only here that all four *domestic* alleles were present (Table 2). In the Andalusian sample ($H_R = 0.73$) not more than 7 of the 11 alleles defined in Portugal were present. In the Azorean sample this was not more than four ($H_R = 0.65$). In Table 5 we show the within-locality correlations (*i.e.* the fraction of total variance due to differences between localities) and the χ^2 statistics for each allele.

The *b* locus gene frequencies of the rabbits raised on local farms were typical for domestic breeds where *b4* and *b5* are often the only alleles (Table 6). The domestic rabbits from Portugal and the Azores, unlike the wild ones, showed no other reactions than "complete" identity reactions when tested with *b* locus antisera, behaving in this respect exactly as rabbits from outside Iberia (domestic or wild). The samples of the Azorean wild rabbits differed from those from the continent by the absence of any *b6* or *b9* reactivity (neither 1, *P*, nor *W*). While a majority of the sera crossreacted with the anti-*b5* antiserum, no *b5* identity reaction was noted. Thus *b4* was the only *domestic* allele present. The sample from Flores did not have a single *b* locus gene in common with domestic breeds. The Azorean sample might be more similar to the Andalusian sample than to any of the Portuguese samples. Different types of tree analyzes based upon NEI's or ROGERS' genetic distances derived from the data in Table 2 clustered the Azorean and Andalusian samples together (not shown). However, such analyzes are highly presumptuous, as the actual identity between similar crossreacting serotypes could not be assessed.

The values of θ and F_{ST} measure the genetic differences between populations. The values in Tables 4 and 5 indicate that the genetic differentiation was

TABLE 2

***b* Locus gene diversity within localities and their means**

Local	Sample sizes	Allotype (%)											Heterozygosity			
		1000 *b4*	*0100* *b5*	*0010* *b6*	*0001* *b9*	*P000*	*0P00*	*00P0*	*000P*	*PP00*	*0P10*	*P0P0*	H_S	D_{IS}	χ^2/d.f.	σ
Portugal																
BR	1		50.0			15.7				34.3			0.61	−0.39	0.3	0.09
VI	5	20.0		10.0	30.0		30.0			10.0			0.76	−0.04	0.6	0.56
ME	7		50.0	28.6		2.2			14.3	4.9			0.65	0.07	0.2	0.12
AN	8	37.5		31.2					25.0	6.2			0.70	−0.30	0.7	0.38
LE	19	8.9	5.6	5.3	5.3	7.4	19.8		2.6	45.1			0.73	0.04	0.9	2.63
ID	11		0.1	9.8	9.1		15.8	30.7		25.0		9.5	0.79	0.03	0.8	0.68
SA	3				33.3		50.0	16.7					0.61	−0.06	0.4	0.22
ST	21	18.8		1.2	7.1	1.0	23.4			29.9	18.6		0.78	0.03	0.7	0.62
SS	9			16.1	22.2	0.1	33.9			26.8	0.8		0.74	−0.17	0.4	0.24
BO	8		6.3			42.5	6.2		6.3	16.1	22.8		0.73	−0.04	0.2	0.13
VV	36	13.5	4.2	27.8	16.8	11.2	8.2		6.8	6.6		4.8	0.84	0.07	0.7	0.79
MO	9	6.3	20.7	11.1	22.2	20.6	1.7		5.6	6.3		5.6	0.84	−0.14	0.5	0.65
RM	33	3.0	17.0	12.1	10.0		31.4		3.7	18.3		4.5	0.81	−0.04	0.5	0.62
BE	7	7.1		14.3	7.1		26.9			30.3		14.3	0.79	−0.11	0.5	0.79
PO	5		30.0	20.0		50.0							0.62	−0.18	0.2	0.09
SI	5	10.0		30.0		14.5			10.0	35.5			0.74	0.05	0.5	0.32
Las Lomas																
L2	38	1.3		9.2	5.3	38.8	22.2			21.8	1.4		0.74	0.00	0.3	0.26
L3	19	2.8			2.6	22.0	44.6			27.9			0.67	−0.03	0.8	0.44
Azores																
AF	60					50.0	34.1			15.9			0.61	0.00	0.0	0.00
AM	57	6.4				30.6	49.3			13.7			0.64	0.00	0.0	0.02
AT	53	14.8				14.2	57.9			13.1			0.61	0.00	0.5	0.20
Means per region																
Portugal	187	9.2	8.6	14.4	10.6	7.7	17.3	2.1	4.6	19.2	3.2	3.0	0.77	−0.02		
	16	7.8	11.5	13.6	10.2	10.3	15.5	3.0	4.6	18.5	2.6	2.4	0.73	−0.07		
Las Lomas	57	1.8		6.0	4.4	33.3	29.7			23.7	1.0		0.72	−0.01		
	2	2.1		4.6	3.9	30.4	33.4			24.9	0.7		0.71	−0.02		
Azores	170	6.7				32.4	46.7			14.1			0.62	0.00		
	3	7.1				31.6	47.1			14.2			0.62	0.00		
Total means																
Regions	3	5.6	3.8	6.1	4.7	24.1	32.0	1.0	1.5	19.2	1.1	0.8	0.69	−0.05		
Subdivision	21	7.2	8.7	10.8	8.1	15.3	21.7	2.3	3.5	18.5	2.1	1.8	0.71	−0.06		
Individuals	414	7.1	3.9	7.4	5.4	21.3	31.0	0.9	2.1	17.8	1.5	1.4	0.70	−0.01		

Allele frequencies and heterozygosity levels (H_S) were estimated from phenotype data (Table 1) for each subdivision (locality) and their means calculated. The χ^2 statistics divided by the degrees of freedom (d.f.) test the hypothesis $D_{IS} = 0$, thus that the observed number of phenotypes are the expression of the proposed alleles at Hardy-Weinberg equilibrium. σ is the standard deviation of the components of χ^2 values.

some two times larger between localities of Portugal than between islands of the archipelago. Note that the estimate of θ following COCKERHAM (1973) makes an ample correction for sampling error (*cf.* the negative value of θ for *PP00* between islands in Table 5). It is interesting that the most pronounced interinsular differences were found for the alleles *1000* and *P000*, which are serologically similar.

DISCUSSION

The *domestic b* locus alleles were present in the common ancestors of modern Iberian and western rabbit populations: Modern wild rabbit populations of the species *Oryctolagus cuniculus* may descend from aboriginal wild rabbits and/or from introduced wild or feral rabbits (free ranging animals derived from domestic breeds). Rabbits were present on the Azorean islands at least since the 15th century at the onset of colonization by Portuguese settlers (CHAVEZ 1911). Populations from western Europe (*i.e.*, north of the Pyrenean mountains) and the domestic races derived from them, are believed to originate from the Iberian peninsula (LOPEZ-MARTINEZ 1977). Rabbits were introduced in France and the British islands as semi-domesticated animals after the Roman Conquest and later to other parts of the world (THOMPSON and WORDEN 1956; FENNER and RATCLIFF 1965). The evidence for an Iberian origin of what will be called here the "western" rabbits is poor, however, and other

TABLE 3

NEI's distribution of gene diversity

	H_I	H_S	H_R	H_T
un	0.78	0.71	0.83	0.86
wt	0.71	0.70	0.76	0.81
	D_{IS}	D_{SR}	D_{RT}	D_{ST}
un	−0.06	0.12	0.03	0.15
wt	−0.01	0.06	0.04	0.11
	F_{IS}	F_{SR}	F_{RT}	F_{ST}
un	−0.07	0.14	0.03	0.17
wt	−0.02	0.08	0.06	0.13

The mean heterozygosity levels H are shown for hierarchical grouping of 828 genes into 414 individuals (H_I), 21 localities (H_S) and 3 regions (H_R). Means were calculated either weighted (*wt*) or unweighted (*un*) by number of individuals within the samples. D and F values express, respectively, the contribution and the relative contributions of differences between subdivisions to the total diversity.

TABLE 4

Genetic differentiation within regions

	H_R		F_{SR}		
Region	*un*	*wt*	*un*	*wt*	θ_w
Portugal	0.88	0.87	0.166	0.115	0.099
Las Lomas	0.73	0.74	0.032	0.027	
Azores	0.65	0.65	0.049	0.052	0.049

The mean heterozygosity within regions H_R and the fraction of it due to interlocality differences within regions (F_{SR}) are shown for each region separately, weighted (*wt*) and unweighted (*un*). REYNOLDS' θ_w is an average over alleles of COCKERHAM's θ values from Table 5, weighted by gene frequencies.

TABLE 5

COCKERHAMS θ for Portugal and the Azorean islands

Allele:	θ	χ^2/d.f.	Allele frequency (%)
Portugal: d.f. = 15			
1000	0.088	2.7	9.0
0100	0.179	4.7	8.2
0010	0.083	2.7	15.0
0001	0.057	2.0	10.6
P000	0.211	5.4	8.9
0P00	0.104	3.3	18.6
00P0	0.298	7.2	2.4
000P	0.058	1.9	4.7
PP00	0.112	3.4	16.9
0P10	0.179	4.6	2.5
P0P0	0.025	1.1	3.3
Mean	0.127		9.1
σ	0.077		5.5
Azorean isles: d.f. = 2			
1000	0.080	10.7	7.0
P000	0.136	15.7	31.7
0P00	0.057	2.3	47.2
PP00	−0.002	0.4	14.1
Mean	0.068		25.0
σ	0.049		15.6

θ is the correlation between pairs of genes from different individuals within the same locality and is analogous to F_{ST}, corrected for variance due to sampling error. The values displayed and their χ^2 statistics are weighted by sample size. The allele frequencies shown were obtained by considering the sample region as one panmectic population. They were used to initiate the reiteration procedure for the frequency estimates in subdivisions, displayed in Table 2.

origins have been proposed, *i.e.*, southern France, where rabbits might have survived the last glaciation (BEAUCOURNU 1977; BODSON 1978). Taxonomists have classified the rabbits from the Mediterranean region as a subspecies (*O. cuniculus algirus*, Lastaste) to be distinguished from the rabbits from western Europe (*O. cuniculus cuniculus*) (LOPEZ-MARTINEZ 1977). Recent analyzes of mitochondrial DNA revealed differences between western and Andalusian wild rabbits compatible with a divergence time of more than one million years (BIJU-DUVAL *et al.* 1990). This would indicate that the domestic rabbits were not directly derived from an Iberian stock.

In 40 rabbits from the Mediterranean island Zembra (Tunisia) not a single *domestic b* locus allele was found (BENAMMAR and CAZENAVE 1981). This is also what we observed on Flores, and is similar to what we found on the other Azorean islands, where only one *domestic* allotype was observed. If the *domestic* alleles would appear to be equally rare or absent on the Iberian peninsula it would become very unlikely that the ancestors of domestic rabbits could have occupied this area in historical times. BENAMMAR and CAZENAVE (1984) reported in a sample of 29 rabbits from Portugal and 18 from Northern Spain, the presence of the *domestic b* locus alleles, at frequencies between 1% and 20%. Our data substantiate and extend this observation: it is thus possible that these alleles may have persisted independently in Iberian and western populations since they became separated.

However, our data also show that in Portugal and on the Azorean islands, domestic farm rabbits constitute, as expected, a reservoir of *domestic b* locus alleles (Table 6). Although unlikely in view of the apparent impossibility to cross domestic with wild Iberian rabbits under natural conditions (N. FERRAND, unpublished observations), gene flow of domestic origin into the gene pool of the wild rabbits can not be excluded. On the Azorean islands only one of the *domestic* alleles was found in wild populations. It was the type which is preponderant in domestic breeds (*i.e.*, the *b4* allele) but which appeared to be no more frequent than any of the other *domestic* alleles in wild Iberian rabbits, the putative ancestors of the Azorean populations (Tables 2 and 6). On Flores, which, due to its extremely western situation in the archipelago was the last island to be colonized by Portuguese settlers, no domestic genes were observed. Thus, on the Azorean archipelago, the distribution of *domestic b* locus genes corresponds to what might be expected if their pres-

TABLE 6

***b* locus gene frequencies in wild, feral and domestic European rabbits (*O. cuniculus*)**

		Allotype (%)										
	Number	*1000* *b4*	*0100* *b5*	*0010* *b6*	*0001* *b9*	*P000*	*0P00*	*00P0*	*000P*	*PP00*	*0P10*	*P0P0*
Wild rabbits												
Portugal	187	9	9	14	11	8	17	2	5	19	3	3
Andalusia	57	2		6	4	33	30			24	1	
Azores	170	7				32	47			14		
Australia[a]	632	62	35		3							
Australia[b]	914	71	27		2							
G. Britain[b]	302	62	23		14							
C. Europe[b]	323	57	37		5							
France[c]	232	52	34	2	11							
Feral rabbits												
Tasmania[a]	49	63	33		4							
Macquerie[a]	128	40	60									
Kerguelen[d]	1200	99	0.1									
Domestic rabbits												
Portugal	21	90	10									
San Miguel	2	75	25									
Belgium[e]	180	81	17		2							
France[c]	1164	86	10	4	0.3							
Australia 1[a]	72	86			14							
Australia 2[a]	24	67	33									
Australia 3[a]	37	93			7							
Australia 4[a]	55	88		12								

Feral rabbits refer here to descendants of domestic rabbits released on rabbit-free islands. Data are from: [a] CURTAIN, WOOD and SOBEY 1973; [b] VAN DER LOO *et al.* 1987; [c] CAZENAVE *et al.* 1987; [d] W. VAN DER LOO and C. P. ARTHUR, unpublished results; [e] C. HAMERS-CASTERMANS unpublished results; *italics*: this paper.

ence in wild populations resulted from admixture with more recently introduced domestic breeds. Before concluding that the *b4* allotypes in wild Azorean populations descend from a common ancestor of western and Azorean rabbits, the hypothesis of horizontal gene flow should be excluded by an appropriate method.

In contrast, the allele distribution on the Iberian peninsula reported here is in strong support of an Iberian origin of the *domestic b* locus alleles. Indeed, the present study confirms not only that all alleles found in domestic breeds are present in wild Iberian rabbits but reveals that they occur at similar gene frequencies, ranging around 10%. Evenly distributed frequencies are predicted on theoretical models that tend to explain maintenance of high allele diversity (LI 1978; LEWONTIN, GINZBURG and TULJAPURKAR 1978; see also below). Such narrow frequency spectrum is, however, not expected when the genetic overlap was due to gene flow from domestic breeds, where alleles display a pronounced and systematic frequency hierarchy, with the *b4* allele being abundant and two others alleles, *b9* and *b6*, rare to very rare (Table 6). Clearly, the situation observed can not be explained by gene exchange between wild and domestic rabbits. Also the fact that in the Iberian rabbits more allotypes exists than in the western rabbits is in perfect agreement with an Iberian origin of the latter. The existence of a large number of *b* locus alleles was indeed anticipated from of the extreme differences between alleles, while the loss of alleles can be explained by founder effects (*cf.* CAZENAVE *et al.* 1987).

To summarize, it appears that at least 11 distinct *b* locus alleles are maintained in the Iberian peninsula at roughly similar gene frequencies. They include all four allotypes found in western populations and probably also all those present in Azorean rabbits, which obviously can not be derived from western rabbits. Western and the Azorean rabbits could therefore descend from an Iberian stock. The persistence time and the coalescence time (see APPENDIX) of *b* locus alleles should therefore be longer than the divergence time between Iberian and western rabbits (*i.e.*, between *O. c. cuniculus* and *O. c. algirus*). This time remains to be determined, but might be as long as long 2 million years (BIJU-DUVAL *et al.* 1990).

Gene diversity: Although the serological approach used here does not warrant a full resolution of the allele diversity, we found that a majority of rabbits were heterozygous at the *b* locus and that the total gene diversity exceeded 80% in the sample under study (see APPENDIX). Different factors contribute to this high genetic variance: the large number of alleles, their even frequency distribution and the interlocality

variances in frequency. Such high levels of gene diversity are exceptional and point to processes favoring diversity at the population level (SINGH and RHOMBERG 1989). They are likely to depend upon strong selective interactions as may exist between parasite and host (BREMERMANN 1980; HAMILTON 1982; DAMIAN 1987). The rational of genetic variation at the constant regions of antibodies as an immune defense strategy was outlined elsewhere (HAMERS, VAN DER LOO and DE BAETSELIER 1986; VAN DER LOO 1987). It is not certain whether a "neck and neck race" between parasite and host is a prerequisite for the *b* locus divergence (*i.e.*, whether the rate of pathogen change is boosting the rate of deterministic change in the host and *vice versa*) as proposed in some models. TAKAHATA and NEI (1990) have recently discussed how the evolution of the MHC polymorphisms can be explained by more conventional models such as overdominance or frequency dependent selection. As significant heterozygous excess at the *b* locus has been reported in wild populations, where only two or three alleles were present (VAN DER LOO *et al.* 1987; VAN DER LOO 1988) it is likely that the high gene diversity observed in the Iberian populations is due to overdominance. The model presented in TAKAHATA (1990) suggests that even with weak balancing selection more than ten alleles can be maintained over long periods of time (more than 1 million years). An analysis of the available data on the *b* locus polymorphism in light of the Takahata and Nei model will be presented elsewhere.

On the Iberian peninsula the average *b* locus heterozygosity approaches 90% (80% within localities), which can be compared with the heterozygosity levels at MHC loci which were estimated at 80%-90% in human populations (KLEIN 1986) and 65% in wild mice (NADEAU *et al.* 1988). We were careful to take into account only very obvious serological differences making an overestimation of gene diversity very unlikely. Indeed, different alleles can express serotypes which under the present methodology failed to be distinguished. Even in domestic breeds, the *b4* allotype comprises two allelic "variants," which differ at two amino acid positions but which display a same serotype if tested with current anti-*b4* antisera (KELUS and PERNIS 1971; VAN DER LOO, HAMERS-CASTERMANS and NAESSENS 1975; SOGN and KINDT 1976, DEKEGEL *et al.* 1981). It should therefore be emphasized that the number of alleles relevant to the mode of selection concerns only alleles distinguished by the selection factors. Thus, counting alleles by serology (*i.e.*, by their ability to elicit an antibody response in others members of the population) could be appropriate in this particular context. Sequence comparisons will be very informative by allowing the estimation of the number of "neutral" mutations accumulated during the history of one particular allotypic lineage (*cf.* TAKAHATA and NEI 1990). The potentials of serological analysis in population genetics of immunological loci should, however, not be diminished.

Distribution of allele frequencies: The serological reagents used in this study should estimate the distribution of the *domestic* types quite accurately. Within Portugal, the differences in mean frequencies between the *domestic* alleles were not significant ($\chi_3^2 = 2.3$, $P = 0.1$). For the other alleles we can only advance minimal estimates of diversity. Our data strongly suggest that none of the eleven alleles exceeds 20% of the gene pool, with the standard deviations of frequencies estimated to be less than 6% (Tables 4 and 5). Theoretical considerations suggest that overdominance can only maintain many alleles in populations if it is highly symmetric (LEWONTIN, GINZBURG and TULJAPURKAR 1978). Symmetric overdominance describes situations where the different alleles are exposed to heterotic selection forces of similar strength. This may well be the case for alleles of Ig loci, where "diversity" could be the key issue. Therefore similar average frequencies of the *b4*, *b5*, *b6* and *b9* alleles are not unexpected if these alleles were maintained by overdominant selection. As the average frequency of the *domestic* alleles is about 10%, the number of alleles expected under symmetrical selection would be ten, which is in close agreement with the number of alleles (11) distinguished here by analyzing the serological crossreactions.

The frequency distribution of *b* locus alleles in western populations is, on the contrary, strongly J-shaped (Table 6). There are different lines of evidence suggesting that the systematic hierarchy in gene frequencies ($p_{b4} > p_{b5} > p_{b9}$) observed throughout the recent range and, by consequence, the low level of their variance among major geographical areas, is due to selection (HERD and EDMONDS 1977; VAN DER LOO 1987). An obvious question is wether the distorted frequency distribution and the limited frequency variances in western populations could be causally related to the loss of an important fraction of the original allele diversity. In the present context it seems interesting that on the Azores, where not more than four of the eleven alleles defined on the peninsula are represented, the interinsular variation seems to be merely limited to the replacement of the *b4*-like *P000* allotype by the *b4* allotype. Comparisons between the cross-reactions of the Azorean samples confirmed the restricted number of serotypes (not shown). The relatively high degree of genetic homogeneity between these island populations is surprising ($F_{st} = 0.05$) considering that they could be separated for more than 500 generations (CHAVEZ 1911) and that their mean effective population sizes should be smaller than on the continent (due to strong founder effects). An

understanding of the forces maintaining the *b* locus allele frequencies in western and Azorean populations should clearly benefit from insights obtained from studies in the original distribution range. Conversely, the small number of well characterized alleles in the western rabbits (and by consequence the much higher proportion of homozygotes), could facilitate the study of these selection forces (*cfr.* VAN DER LOO *et al.* 1987; VAN DER LOO 1988).

Concluding remarks: Rabbits (*O. cuniculus*) thrive in most parts of the world and have been reported on more than 5000 islands (FLUX and FULLAGAR 1983). In many regions their population densities have significantly been affected by a viral disease, myxomatosis (FENNER and RATCLIFFE, 1965; ROSS and TITTENSOR, 1981). Complex polymorphisms of the immunoglobulin constant region loci are known in most other mammals studied (MAGE *et al.* 1973), and an evaluation of the adaptive value of this type of variability should be of general interest. In the field of population genetics and evolutionary theory the rabbit Ig polymorphisms offers a model system which might be exceptionally informative. A comparative study on the population immunogenetics of the European rabbit in its ancient and recent areas of distribution should allow a comparison of the effect of the selection forces not only in a variety of ecological and epidemiological environments but also in different genetical contexts. The *b* locus allele diversity and its distribution, as reported here for Iberian populations, are in good agreement with predictions of population genetical theory for systems that maintain extensive genetic diversity in populations. This substantiates considerably the evidence that the *b* locus divergence is the outcome of balancing selection and strongly suggests that alleles have persisted in populations at least since the separation of the ancestors of Iberian and domestic rabbits. Recent theoretical developments in allelic genealogy provide an analytical framework which seems to fit phylogenetical and population genetical observations on the MHC loci, including the trans-species polymorphisms and the increase in evolution rates (TAKAHATA 1990): the rabbit *b* locus diversity, which shows important similarities but also some striking differences with that of the MHC loci, could allow a more general appraisal of these models, thereby contributing to our understanding of the impact of parasite-host interactions on the origin and maintenance of population variability and how this relates to evolutionary change.

We thank MASADO CRUZ and ANTONIO AMORIN for offering facilities in their laboratories at the University of Porto and for stimulating discussions. GIL CARVALHO provided the Azorian samples. ANN MCBRIDE assisted us generously and JOSE RAMON MORA FIGUEROA and his son FERNANDO offered loding and logistics during the collection of the Andalusian rabbits. CECILE HAMMERS-CASTERMAN and RAYMOND HAMERS played an essential role in the development of the antisera and of the project in general. This work was partially financed by a grant of the Belgian National Science Foundation (NFWO-FNRS) to W. v.d.L., who is research assistant at this insitution.

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Communicating editor: W.-H. LI

APPENDIX

Note on estimation of b locus coalescence time: Serological data on different leporid species have indicated that the *b* locus polymorphism predates the speciation of modern leporids (RODKEY and CONRAD 1972; LANDUCCI-TOSI, TOSI and PRESSAMON 1973; LANDUCCI-TOSI, MAGE and TOSI 1976; VAN DER LOO

1975; VAN DER LOO, HAMERS-CASTERMANS and HAMERS 1976; SMITH and MANDY 1978; VAN DER LOO *et al.* 1980; W. VAN DER LOO, unpublished results). However, according to VAN DER LOO (1975), a more refined serological analysis suggested that, despite the extensive interallelic differences in protein structure, the divergence of the *domestic b* locus alleles should be more recent than the separation between *Sylvilagus*, *Lepus* and *Oryctolagus* species. The *b* locus coalescence time should then be smaller than 6 million years (LOPEZ-MARTINEZ 1977). This seems to be corroborated by the strong homologies between noncoding parts of these alleles and the paucity of synonymous substitution. The mean allele coalescence time (T_C) depends upon important population genetical parameters (TAKAHATA and NEI, 1990), some of which are difficult to assess (*i.e.*, the phylogenetic effective population sizes and selection forces). Our understanding of the evolution of the *b* locus polymorphism might clearly benefit from a reliable estimate of the its mean coalescence time T_C. It was therefore important to ascertain that the *domestic* allotypes occurring in wild Iberian populations are indeed the reminiscence of aboriginal allelic lineages, as this should allow the definition of a lower limit of the coalescence time of the *domestic b* locus alleles.

Note on the estimation of gene diversity: By initiating the reiteration procedure with the mean frequencies we tend to minimize interlocality variance, which is one of the components of the total heterozygosity. Thus on Flores, where only three phenotypes (P000, 0P00 and PP00) were found, there might only be two alleles *P000* and *0P00* in stead of three as we assume. On the total sample, the existence of a *PP00* type had to be postulated in order to explain the phenotype distribution in Portugal with a minimal number of alleles. We estimate therefore the minimal genetic distance between the two areas.

On the other hand, we might overestimate gene diversity by assigning more alleles than necessary in particular samples. This may occur if there is an excess of heterozygotes which can be reduced by postulating the existence of an allele which is not necessarily present. Thus on Flores, H_S will be 0.49 if we postulate two alleles rather than 0.61 with three alleles (Table 7). However these differences are merely due to a strongly negative D_{IS} (heterozygous excess) in the two allele model while D_{IS} is zero in the three alleles model. The estimation of H_I is similar in both cases (Table 7) and is clearly not overestimated by postulating three alleles. Thus our estimate of H_S is biased toward H_I, which estimates the actual fraction of heterozygotes.

Conversely, assigning fewer alleles than actually exist does not lead to an overestimation of H_T. We consider all (P100) rabbits as heterozygous *P000/0100* although some might be homozygous *P100*, a putative allele not recognized in our procedure. If so we would overestimate H_I but still obtain a conservative estimate of H_S (and H_T) which increases with increasing numbers of alleles. The possible overestimation of H_I in the our model would be compensated by an underestimation of the sources of diversity due to differentiation within localities (D_{IS}) or/and between localities (D_{ST}). On the extreme hypothesis that all rabbits in Portugal were homozygous ($H_I = 0$), there would be 33 alleles and H_R would equal 0.95, a value not so different, but clearly not smaller than the 0.87 value found with eleven alleles at near Hardy-Weinberg equilibrium (Table 7).

The estimation of NEI's total gene diversity from the phenotype data is in fact quite robust and conservative. It relies merely upon the assumption that different serological patterns do indeed reflect differences in genotypes. That this is the case was established in numerous studies, including studies in other leporid species (*Sylvilagus bachmani* and *S. floridanus*) where the binomial distribution or/and the Mendelian inheritance of crossreaction patterns similar to those here described, was confirmed (VAN DER LOO *et al.* 1980; W. VAN DER LOO and C. P. ARTHUR, unpublished results). The most direct and convincing evidence comes from the work carried out by the group of the late J. OUDIN (Institut Pasteur, Paris), where crossreacting *b* locus alleles from the island Zembra were introduced by artificial insemination into domestic stocks (reviewed in CAZENAVE *et al.* 1987).

TABLE 7

Estimates of heterozygosities H_S and H_I under different hypotheses

Hypothesis		H_S	H_I	D_{IS}	F_{IS}
Flores					
2 alleles	$D_{IS} \approx 0$	0.49	0.63	−0.14	−0.29
3 alleles	$D_{IS} \approx 0$	<u>0.61</u>	0.61	0.0	0.0
Portugal (undivided)					
11 alleles	$D_{IS} \approx 0$	<u>0.87</u>	0.81	0.06	0.07
33 alleles	$H_I \approx 0$	0.95	0.00	0.95	1.00

By allocating a number of alleles such that D_{IS} is minimal, the reiteration procedure estimates heterozygosity levels (H_S, underlined) that are in general not higher than what would be estimated (H_I or H_S) by adopting a different hypothesis.